Assessing the Efficacy of Enzyme Assays as a Soil Health Indicator on Diverse Long-Term Agronomic Plots

Honors Research Thesis

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ABSTRACT

To guide land managers and policy makers, soil health indicators are needed that are temporally sensitive and reflect the ability of soils to deliver ecosystem services. This study investigated the ability of two enzyme assays, β-glucosidase (GLU) and arylsulfatase (ARS) to detect soil management treatment effects from three long-term research sites (>17 years) in North Carolina that had distinctly different soils and environments (Coastal Plain, Piedmont, and Mountain soils). Soil was collected in the 0-15cm depths and air-dried before running the assays. For both enzyme assays, there were significant differences between treatments for all three physiographic regions compared to Cornell Soil Health Assessment and Haney Soil Health Test which detected treatment effects at only one of three sites. Enzyme assays ranked treatments with known best management (no-till, organic) higher than intensive tillage and chemical treatments for all three sites. At the Mountain site, ARS and GLU had over 2.5 times the activity in the no-till organic (NTO) treatment compared to the conventional tillage chemical (CTC) treatment, compared to CASH which gave a score of 55 for NTO and 44 for CTC. GLU showed no significant difference between treatments, but when controlled for sand content (GLU_S) showed significant difference with two groupings. ARS had a stronger correlation to fatty-acid methyl ester (FAME) biomarkers than GLU, and both were overall weakly correlated to FAME ($r < 0.34$). Overall β-glucosidase and arylsulfatase were able to distinguish between management systems and consistently rank soils with improved management as having higher enzyme activity per unit sand.

INTRODUCTION

SOIL HEALTH INDICATORS

Soil is a vital natural resource that provides a suite of ecosystem services and is critical in global biogeochemical cycling. Sustainable soil management and metrics that can inform

management decisions and track progress are critical to conserving soils under intensive agriculture and a changing climate. Analytical metrics that look at the holistic soil ecosystem are commonly referred to as soil health metrics. Soil health has been defined by the USDA-NRCS as the capacity of a soil to function as a vital living ecosystem (2015). This definition has been expanded to include soil resiliency in the face of disturbance (Doran & Parkin, 1994). Soil testing for nutrient status is well established, but it does not measure the ability of a soil to provide ecosystem functions such as phytopathogen suppression, resistance to erosion, or carbon sequestration. The quantification and interpretation of dynamic soil properties and ecosystem functioning across the array of soil types remains a point of debate among soil scientists (Roper et al., 2018; Van Es & Karlen, 2019).

To tackle this issue, two commercial labs of note have emerged with pioneering quantitative soil health assessments: Cornell Soil Health Assessment (CASH) and Haney Soil Health Test (HSHT). CASH is used by the US Department of Agriculture and measures a suite of physical and chemical properties and one biological property (respiration) to calculate a soil health score (Gugino et al., 2009). CASH has been in place since 2006 and, according to its website, has conducted over 10,000 soil health tests commercially and for institutions such as Soil Renaissance, Soil Health Partnership, and the USDA-NRCS Soil Health Division (Cornell Soil Health Team, 2018). The HSHT is a measurement of respiration after a soil has been air-dried and rewetted along with various carbon (C) and nitrogen (N) assessments and no physical property tests (Ward Laboratories, Inc., 2018). Soil health is difficult to quantify because the vast array of soil types is variably influenced by the five soil forming factors: climate, biota, parent material, relief, and time. Management is one of many variables, including the soil forming factors, that contributes to soil ecosystem functioning. Regardless, it is critical that soil health

tests are sensitive enough to management effects to differentiate between treatments in order to inform practical land use recommendations to managers.

Roper and team (2017) tested the ability of CASH and HSHT to differentiate among management in three long-term agronomic research sites. These sites had a range of tillage, crop rotation, and chemical or organic practices. However, the results from CASH and HSHT showed few significant effects on soil health scores due to management. More importantly, soil management treatments known to improve soils did not get high scores (Roper et al., 2017). The results for CASH were contested in 2018 by Van Es & Karlen, saying that individual soil health indicators were able to differentiate between land management regimes. However, the 2019 rebuttal by Roper and team emphasized that overall soil health scores did not show significant differences between treatments and had little correlation with yield ($R^2 = 0.13$) (2019).

This indicates the need for further research into soil health metrics that meet the following criteria. 1) Soil health metrics should reflect the ecosystem functioning of the soil, providing an integrated measurement of the biotic, physical, and chemical properties of soil. In order to be practical, 2) metrics must be able to distinguish between management regimes and 3) detect management changes on the order of 3-5 years. This eliminates many soil properties that may take decades (e.g. soil organic matter) or geological time to change. 4) Metrics should be economically viable on a commercial scale. Lastly, 5) results of soil health tests must be calibrated and interpretable across soil types. This problem is the major limitation of all proposed soil quality indicators because most soil health indicators vary more by soil type than subtle soil management effects.

ABIONTIC ENZYME FUNCTION IN SOILS

One proposed soil health metric that meets the above requirements is the enzyme assay (Dick, 1994). To assess ecosystem functioning, many biological parameters have been suggested including respiration and microbial biomass among others. However, many biological indicators fluctuate on a seasonal basis along with changes in rainfall and temperature. This variability makes it difficult to calibrate and interpret results year to year. In contrast, enzyme activity is a biological measurement that has the potential to provide an integrative, long-term assessment of the soil biota without seasonal variability (Acosta-Martinez et al., 2013; Bandick & Dick, 1999).

For many soil enzymes, the activity measured is a combination of activity from enzymes associated with viable cells (e.g. internal or cell surfaces) and enzymes stabilized in the soil matrix, which are known as abiontic enzymes. These abiontic enzymes can remain catalytic (Dick, 1994). Enzymes can enter the soil matrix through several pathways. Upon cell death, enzymes are released through cell lysis or complexed in cell debris. Additionally, fungi, bacteria and plants excrete extracellular enzymes into soil solution that hydrolyze large macromolecules to be taken up into the cell. Most enzymes entering the soil are rapidly degraded, but some are stabilized in the soil matrix through adsorption to clay surfaces or organic matter (Burns, 1978). This strong adsorption protects enzymes from degradative proteases. Stabilized enzymes remain catalytic with typically 40-60% of the activity associated with the abiontic form of many enzymes (Knight and Dick, 2004; Vallejo et al., 2010). Enzymes with a significant amount of abiontic activity would be good candidates for soil health indicators because they accumulate more slowly and reduce seasonal variability (Dick, 1997). Screening for enzymes that are both sensitive for detecting the effects of land management or degradation and low seasonal variability showed that β-glucosidase (GLU) and arylsulfatase (ARS) meet this criteria (Bandick & Dick, 1999). Subsequent research on a variety of soils, agricultural management systems, and

environments further confirmed the potential of these assays as an indicator (Ndaiye et al., 2000; Knight & Dick, 2004; Balota et al., 2004; Acosta-Martinez et al., 2013, 2019; Carlson et al, 2015; Higusa et al., 2004; Mendez et al., 2019; Vallejo et al., 2010).

β-glucosidase is an extracellular enzyme that catalyzes the breakdown of cellulose into glucose (Turner et al., 2002). Arylsulfatase hydrolyzes ester sulfate bonds that release plant available inorganic SO_4^{2-}. Activities of ARS and GLU are useful indicators because they relate key soil functions (S and carbon cycling) and represent the cumulative integration of the microbial community structure at the time of sampling (Sun et al., 2014). Microorganisms depend on optimal soil habitat conditions such as soil structure, aeration, water holding capacity, and availability of energy resources (e.g. soil organic matter). These same soil properties are related to optimal plant growth and resistance to soil erosion.

ENZYME ASSAYS AS SOIL HEALTH INDICATORS

Enzyme assays have been shown to meet the requirements for an effective soil health indicator. Management systems that protect and improve soils through less disturbance and greater C inputs will stimulate microbial populations and enzyme production. Therefore, it seems plausible that practices that promote aggregation and organic matter accumulation would also promote stabilization and protection of abiontic enzymes in the soil humic-matrix. Enzyme activity should provide useful information on whether soil management is promoting soil organic matter development long before measurable changes in organic C can be detected. Moraes Sa and researchers (2018) showed the strong correlation (r = 0.90) of enzyme activity to soil organic C content.

Sensitivity to land management

Soil enzyme activities hold potential as early and sensitive indicators of soil ecological stress or restoration (Bandick & Dick, 1999; Ndaiye et al., 2000; Hinojosa et al., 2004; Acosta-Martinez et al., 2019). Land use influences biotic community composition. ARS activity has been shown to differentiate between forest, pasture, and cultivation management (Vallejo et al., 2010; Moraes Sa et al., 2018). ARS activity decreases after forest and pasture clearing for cultivation and can detect differences in cleared land which has been cultivated or left fallow (Farrell et al., 1994). In agronomic systems, ARS has shown a 215% higher activity in no-till plots compared to conventional tillage (Balota et al., 2004), and this tillage response has been confirmed in various soil types (Acosta-Martinez et al., 2019). Likewise, Bandick & Dick demonstrated the ability of GLU to distinguish field management such as cover cropping and organic amendments to soils. GLU is regarded as one of the most sensitive assays for detecting cultivation intensity (Mganga et al., 2015).

A desirable characteristic of ARS and GLU activities is that they are not suppressed by standard NPK fertilizers (Dick, 1994). Thus, enzymes involved in N or P cycling are not desirable as soil health indicators, specifically those whose products are the same as inorganic fertilizers, namely NO_3^-, NH_4^+, and PO_4^{3-}. For example, phosphatases and aminases are suppressed by a feedback mechanism when their products are in excess (e.g. PO_4^{3-} and NH_4^+) (Dick, 1994). However, GLU has responded positively to fertilization when it leads to increased plant biomass C inputs to soil (Geisseler & Scow, 2014; Kooch et al., 2019). ARS and GLU also regularly demonstrate temporal responsiveness to management changes with ARS even differentiating between restoration forest stands within 4 to 7 years after change in management (Moraes Sa et al., 2018). ARS and GLU have also been shown to respond to other forms of

amendments such as gypsum and lime application, responding positively to a more neutral pH more suitable to plant productivity (Inagaki et al., 2016).

Practical considerations

Since it is insufficient for a soil health indicator to be accurate for use on a commercial scale, practical limitations of cost, interpretability, and scalability must be considered. An important factor is sampling depth. In that regard, Wallenius and team (2011) reported that since soil enzymes originate from the microbial community, the top 15 cm of soils is optimal for depth for measuring microbial properties.

Soil enzyme activities have the potential to overcome a practical problem of nearly all other soil microbial measurements, the requirement for immediate analysis of fresh soil samples or storage at -20 ºC. Air dried soils, however, can be stored at 4 ºC for far longer and do not require immediate analysis. Bandick and Dick (1999) compared management treatments of soil enzyme activity on fresh and air-dried soil, and found that for selected enzymes assays (including GLU and ARS) both pretreatments enabled detection of soil management. They found that although the activity of most enzymes went down some, the ranking of management treatments remained the same between air-dried and fresh moist soil. This is a great asset for commercial and high throughput labs because they routinely use air-dried soil for traditional soil fertility testing. There is a strong body of literature that shows selected air-dried enzyme activities are consistent, reproducible, and quantifiable in detecting land management (Mendes et al., 2019; Hinojosa et al., 2004).

A major limitation of nearly all (if not all) potential measures of soil health is that results vary widely as a function of soil type. This variation can be greater than the effect of land management. Enzyme activities have potential to overcome this by normalizing activity to some

soil texture fraction (sand, silt, or clay) (Knight, 2004; Vallejo et al., 2010). This seems to be most appropriate for enzymes with a high abiontic fraction. This is because abiontic enzymes are largely stabilized primarily in the clay and secondarily in the silt fraction. The objective of this study was to determine the ability of enzyme assays to detect between agronomic management systems in long-term experiments in North Carolina and compare to CASH and HSHT soil health scores at the same sites.

MATERIALS AND METHODS

Sites and Sampling

Soil samples were collected from long-term agronomic research sites representing three eco-regions of North Carolina: Coastal Plain, Piedmont, and Mountain. These sites vary in climate and intrinsic soil characteristics (i.e. texture, minerology, soil series). The Coastal Plain, Goldsboro site, has a Whickham sandy loam (fine-loamy, mixed semiactive, thermic Typic Hapludult) located at the Center for Environmental Farming Systems research farm (35°22’59.9808”N, 78°2’19.6722”W). This experiment has a completely randomized design with three replications following treatments: 1) no-till with chemical inputs (NTC), 2) conventional tillage with chemical inputs (CTC), 3) conventional tillage organic 1 (CTO1), and 4) conventional tillage organic 2 (CTO2). Treatments CTO1 and CTO2 had differed in crop rotation and use of cover crops. This experiment initiated in 1999 or 21 years at the time of soil sampling. The CTO1 has a corn and soybean rotation with winter fallow, and CTO2 has a corn, soybean, and sunflower rotation with rye and leguminous cover crop. Both organic plots received raw poultry litter for their nutrient needs.

The second site is located at the Upper Piedmont Research Station (36°23’2.1372” N, 79°42’6.8436”W) near Reidsville, North Carolina, and was established in 1984, or 36 years at the time of soil sampling. The site has a Toast coarse sandy loam soil (fine, kaolinitic, mesic

Typic Kanhapludult). The experiment had a completely randomized block design with four replications and the following treatments: 1) no-till (NTC), 2) in-row subsoiling in spring (IRS), 3) disking in spring (DS), 4) chisel plowing in fall (CPF), 5) chisel plowing in spring (CPS), 6) chisel plowing with disking in fall (CPDF), 7) chisel plowing with disking in spring (CPDS), 8) moldboard plowing with disking in the fall (MPDF), and 9) moldboard plowing with disking in the spring (MPDS). All treatments used chemical fertilizer and pesticides. The rotation across all treatments was corn and soybeans.

The third site at the Mountain Horticultural Crops and Research Extension Center (35°25’39.126”N, 82°33’24.7068”W), Mills River, North Carolina, was established in 1994 (25 years since the time of sampling) on a Delanco silt loam (fine-loamy, mixed, semiactive, mesic Aquic Hapludult). The experiment had a completely randomized design with five replications and the following treatments: 1) no-till organic (NTO), 2) no-till with chemical fertilizer and pesticides (NTC), 3) chisel and disk tillage with organic management (CTO), 4) chisel and disk tillage with chemical fertilizer and pesticides (CTC), 5) and chisel and disk tillage with no fertilizer or pesticide inputs (CTX). Organic treatments received pelleted poultry litter for their nutrient needs. All treatments had continuous corn until 2013, after which there was a corn and soybean rotation. Further information for all three experiments can be found in Table 1.

Soil samples were collected in November of 2015 for the Piedmont site, and in December of 2015 for the Mountain and Coastal Plain sites. A composite sample was made from 4 to 5 subsamples of each replication using an auger (3 cm diameter) to a depth of 15 cm. In July of 2019, samples were again collected from the three sites using a 2-cm diameter soil probe (15cm depth) to collect 8 to 10 subsamples that were composited. Samples were passed through a 2-mm sieve. About 50 g of soil was incubated at 60 ºC for 24 hrs for textural analysis. Air dried soil

was used for enzyme activities whereas fresh soil was stored at 4 ºC and used for EL-FAME analyses. At time of sampling, the surface soil at the Reidsville (Piedmont) site was exceedingly dry (gravimetric water content < 9%), and the decision was made to re-wet the soil to 1/3 field capacity and incubate for one week at room temperature before returning soils to 4 ºC storage and subsequent use for EL-FAME analysis.

Laboratory Analyses

The enzyme assays β-glucosidase (EC 3.2.1.21 b-D-glucoside glucohydrolase) and arylsulfatase (EC 3.1.6.1 arylsulfate sulfohydrolase) were determined as described by Deng and Popova (2011) and Tabatabai (1994) respectively. However, toluene was not used. Three-gram soil samples were incubated at 37 ºC for 1 hr with appropriate substrate under pH buffered conditions. Two replicates and one control (sample with soil and reagents, but with the addition of substrate following reaction termination) were run for each sample. After incubation, the reaction was stopped using 4 ml THAM (tris-hydroxymethyl aminomethane) pH 12 and 1 ml of 0.5 M $CaCl_2$. The sample was then filtered through Whatman #2 filter paper. The absorbance of the filtrate was measured using a spectrophotometer at 415 nm. The absorbance value of the control was subtracted from both replicates, and the two replicates averaged. Enzyme assays were rerun if variability between replicates exceeded 5%. A calibration curve was developed using standards containing 1, 100, 200, 300, 400, or 500 nmol p-nitrophenol in MUB diluted in a 1:1 mixture of MUB pH 6.0 and 0.1 M THAM pH 12. When absorbance values exceeded that of the highest p-nitrophenol standard solution, the colorimetric solution was diluted to obtain an absorbance within the standard curve.

Soil texture article size analysis was run using the hydrometer method as prescribed by Bouyoucos (1962). After sustained shaking with a sodium dispersion agent, the density of

solution was measured at two time intervals to determine sand and clay percentages. Texture was analyzed on four samples from the Piedmont and Mountain and averaged because each site had the same soil type throughout. The Coastal Plain site had different soil types throughout plots and so texture was analyzed for each sample. Silt was then back calculated. Values for pH were determined via a pH probe in a 1:1 soil to distilled water solution (EPA, 2004).

To determine the soil microbial community structure, EL-FAME (ester-linked fatty acid methyl ester) was run using the method developed by Schutter and Dick (2000). The soil's ester-linked lipids are converted to methyl-esters by alkaline methanolysis with the addition of 0.2 M KOH to 3 g of fresh soil. The sample is then incubated for 1 hr at 37 ºC with intermittent vortexing and then brought to neutrality using 0.1 M acetic acid. The addition of hexane and sustained vortexing transfers the FAMEs from the aqueous phase to the organic phase. The samples were then centrifuged for 20 min at 500 × g to separate the organic phase. This aliquot was evaporated under nitrogen gas to prevent degradation of the FAMEs. The sample is dissolved into a 1:1 solution of hexane-MTBE (methyl tertiary-butyl ether) and detected using a gas chromatography – flame ionization detector (GC-FID) (Agilent 6890, Agilent Inc., Wilmington, DE) equipped with a 25-m HP Ultra-2 column (internal diameter, 0.2 mm; film thickness, 0.33 µm). The GC runs ramped the temperature from 170 to 280 ºC by 4 ºC per min. Between samples, the column was cleaned by holding temperature at 280 ºC for 5 mins. Standards were used to identify individual fatty acids: 37 FAMEs mixture (FAME 37 47885-4; Supelco, Inc), 24 bacterial FAMEs mixture (P-BAME 24 47080-U; Supelco, Inc.), and MIDI standards (Microbial ID, Inc.). Varying concentrations of tridecoanoic FAME (Supelco, Inc.) were used to quantify FAMEs.

Statistical analyses

The SAS Univariate procedure (SAS version 9.3) was used to evaluate the distribution of data. Significant differences between treatments were determined using a one-way analysis of variance (ANOVA) with randomized complete block design. ARS and GLU were normalized before running ANOVA procedures by transformation with square root procedure for GLU and log procedure for ARS. Individual treatment comparisons were made using Duncan's means separation. Comparisons across sites controlling for sand content was done by dividing activity (μg PNP g^{-1} hr^{-1}) by unit sand (%) represented as ARS_S and GLU_S for arylsulfatase and β-glucosidase, respectively. The relationship between FAME biomarker concentrations (nmol g^{-1}) and enzyme activity was determined by simple linear correlation using SAS software.

RESULTS AND DISCUSSION

The three physiographic regions vary in climate, soil forming factors and soil type. The Coastal Plain site has a Wickham sandy loam (Typic Hapludult) and Tarboro loamy sand (Typic Udispamment). These soils are characterized by development in marine and fluvial sediments with relatively high mean annual temperature and rainfall and good drainage. The Piedmont site is classified as a Toast coarse sandy loam (Typic Kanhapludult). This is another well-drained soil formed in high mean annual temperature and rainfall, but the Toast series forms from residuum. The Mountain site soil is mapped as Delanco silt loam (Aquic Hapludult) formed from alluvium. This soil has the lowest mean annual temperature and rainfall of the three sites but is also well-drained.

The Roper paper (2017) extracted humic matter (HM) using an alkaline NaOH solution (Mehlich, 1948) and was highest in the Coastal Plain site, ranging from 0.44% to 0.58%, averaging 0.50%. The Mountain site had the next highest HM ranging from 0.23% to 0.45%, averaging 0.32%. The Piedmont site had the lowest average HM of 0.20% and ranging from

0.10-0.32%. HM was not significantly ($p < 0.05$) affected by management treatment for all experimental sites (Roper et al., 2017).

The North Carolina soils, on average, had pHs ranging from slightly acidic to very acidic (Coastal Plain = 6.3, Mountain = 6.3, Piedmont = 5.9) (Table 1). There was no significant correlation between pH and ARS activity, but GLU had a significant ($p = 0.0314$) positive correlation between activity and soil pH. This is concurrent with previous studies looking at GLU response to more neutral pH (Inagaki et al., 2016). Treatments showed no significant relationship with pH, but there was a trend of lower pH in organically managed soils.

Enzyme Activity

Amendments

Soil amendments of the experimental sites ranged from synthetic fertilizers and pesticides to organic litter and cover crop residue. The Coastal Plain and Mountain experimental sites had various amendment treatments. GLU and ARS activities significantly ($p < 0.05$) increased due to organic fertilizers compared to chemical fertilizers at the Coastal Plain site. The CTC treatment had an ARS of 11.4 µg PNP g^{-1} hr^{-1}, significantly less than the 17.8 µg PNP g^{-1} hr^{-1} of CTO1 and 21.3 µg PNP g^{-1} hr^{-1} of CTO2 (Figure 1). While differences between GLU were not significant, CTC similarly had a lower activity of 18.4 µg PNP g^{-1} hr^{-1} compared to 23.4 µg PNP g^{-1} hr^{-1} of CTO1 and 26.0 µg PNP g^{-1} hr^{-1} of CTO2 (Figure 2). These results are consistent with previous studies where GLU activity increased due to organic N sources over synthetic N (Sharma et al., 2013; Miller & Dick, 1995). Dick and others (1988) investigated various organic and synthetic N fertilizer treatments and found the highest activity of ARS and GLU in soils with manure and organic legume residue additions.

ARS and GLU both had CTO2 as the highest activity at the Coastal Plain site. CTO2 utilized winter cover crops (rye and legumes) compared to CTO1 which had winter fallow. The CTO2 had the highest input of organic C and higher enzyme activities which is consistent with that of Bandick and Dick (1999) with cover crop treatments. Similar studies by Deng and Tabatabai found GLU (1996) and ARS (1997) had their highest activity in treatments with mulch compared to bare soil across a variety of tillage regimes.

The Mountain site showed similar response of GLU and ARS to organic amendments. ARS was 60.4 µg PNP g^{-1} hr^{-1} in NTO compared to 38.0 µg PNP g^{-1} hr^{-1} in NTC. In GLU, the difference was even more pronounced with 48.5 µg PNP g^{-1} hr^{-1} in NTO compared to 21.7 µg PNP g^{-1} hr^{-1} of NTC. In conventionally tilled treatments, the effects were the same with GLU activity for CTO nearly double that of CTC and ARS activity for CTO over double that of CTC. CTX, which did not have organic or synthetic fertilizer amendments, was not significantly different from CTC for GLU activity and had slightly higher ARS activity. For both GLU and ARS, the non-amended control had less activity than the organically amended treatment under the same tillage regime. Responses of higher activity in organically amended plots compared to non-amended plots has been shown for both ARS (Darby et al., 2006) and GLU (Cespedes et al., 2006).

For both sites and both enzymes, organically amended treatments consistently had higher activity than inorganically amended or unamended treatments. This is consistent with research by Carlson and team which compared enzyme activity response to a variety of organic amendments and found all organic amendments resulted in higher activity than the control, unamended plot (2015). Furthermore, both ARS and GLU were able to distinguish the cover cropped treatment with organic amendments from the non-cover cropped treatment with organic

amendments. Previous work found increasing ARS activity with increasing rates of green manure (Ochiai et al., 2008).

Tillage

All three sites had treatments with various tillage regimes. The Piedmont site only had tillage treatments, each combined with chemical methods. At this site, GLU significantly detected differences ($p = 0.0039$) in tillage methods but conservation treatments (NTO and IRS) received lower ranking than more intensive tillage regimes (CPS, CPF, and CPDF). However, a factor that could account for this is that chisel plowing distributes the organic plant matter to about 15 cm and is more intimately in contact with the soil matrix and microorganisms compared to no-till that keeps residue on the surface. Furthermore, GLU activity is stimulated by rapid decomposition (Jian et al., 2016). Thus, in comparing chisel plowing with no-till in samples for the 0-15 cm depth, the chisel plowing could overall stimulate greater production of enzymes involved in organic matter decomposition. This would follow studies on enzyme activity of no-till and conventional tillage which showed that while no-till had higher activity at the 0-5 cm depth, conventional tillage had higher activity at the 5-15 cm depth (Veum et al., 2015). The most intensive tillage regimes (MPDS and MPDF) of the Piedmont had the lowest ranking in ARS and the second and third lowest ranking for GLU activity. GLU activity ranked DS the lowest. This tillage leaves only 16% residue on the surface according to work by Raper (2002).

Furthermore, Yang and Wander (1998) on various tillage systems found aggregate dry mean weight diameter was not significantly different between disk till and moldboard plowing. ARS and GLU ranked CPS and CPDS higher than CPF and CPDF, respectively. This is likely due to the elimination of residue cover with fall tillage which leaves soil bare a significant portion of the year (Nunes et al., 2018). Deng and Tabatabai (1996, 1997) found consistently higher

enzyme activity in mulched systems compared to bare across several tillage regimes. Additionally, tillage systems with residue cover were significantly higher in both ARS and GLU than no-till systems left bare (Deng & Tabatabai, 1996, 1997).

The Coastal Plain and Mountain experiments utilized either no-till (NTC, NTO) or chisel tillage (CTC, CTO). At the Coastal Plain, GLU and ARS activities ranked NTC higher than CTC, although this was only significant in ARS which had 1.8 times the activity in NTC compared to CTC. The Mountain site compared differences in tillage for both organic and chemical systems. Both GLU and ARS had significantly higher activity in NTC compared to CTC. Both enzymes also had higher activity in NTO compared to CTO but this difference was only significant for GLU activity.

Combining no-till with organic input (NTO) had the largest increase in GLU activity, where NTO had 2.5 times greater GLU activity then the intensive tillage of CTC. In ARS, NTO had 2.7 times the activity of CTC. The positive response of GLU and ARS to no-till systems is well documented (Dick, 1992; Eivazi et al., 2003; Balota et al., 2004; Dick, 1984). Landmark work by Gupta and Germida (1988) assessing a range of physical, chemical, and biological properties of cultivated and undisturbed soil found significantly higher ARS activity in undisturbed compared to cultivated soils, especially in the macroaggregate fraction. Recent work by Balota et al. confirms the effect of higher ARS activity in uncultivated soils (2014).

The ability of ARS and GLU to detect subtle management differences such as the presence or absence of cover crops, differences in tillage, and differences in organic vs. chemical treatments makes these enzyme assays promising soil health indicators. Additionally, practices known to improve soil health and ecosystem functioning received higher rankings than practices known to degrade and deplete soil.

Enzyme Activity Standardized for Texture

Standardization procedures for cross-site comparisons of soil health indicators are necessary because nearly all, if not all, soil health indicators vary more as a function of soil type than by soil management effects. This is true for microbial properties including microbial biomass C, soil respiration (Groffman et al., 1996), PLFA profiles (Bossio & Scow, 1998; Ibekwe & Kennedy, 1998), FAMEs (Schutter et al., 2001), and enzyme activities (unpublished data, R. Dick, 2020). Acosta-Martinez and team (2003) found that a sandy clay loam with the same treatment as a loamy sand had over 4 times the GLU activity. Similar work by Lee and others (2007) on ARS found almost 1.5 times the activity in a clay soil compared to a loamy sand soil with the same treatment. In order for soil health indicators to be useful on a national or global scale, standardization of enzyme activity interpretation is critical.

Texture was determined to be a major factor in controlling for enzyme activity due to the significant amount of abiontic enzymes (stabilized but catalytic enzymes in the soil matrix). Clay, and to a lesser extent silt, fractions adsorb and stabilize enzymes on their internal lattice structures and are typically complexed with humic colloids (Boyd & Mortland, 1990; Quiquampoix et al., 2002). In the late 1950s and into the 1960s, studies showed that enzymes can be strongly sorbed by clay which affects their activity, kinetics, and stability (McLaren & Packer, 1970). A number of studies where soils were fractionated for texture and assessed for enzyme activities supported this textural distribution including esterase, carbohydrolase, and urease activities (Haig, 1955; Hoffmann, 1959).

This stabilized enzyme fraction of activity was isolated by various sterilization techniques. This was first done by high-energy electron beams or gamma radiation in the 1950s which utilized ionizing radiation for sterilization of soil (Dunn et al., 1948; McLaren et al., 1957, 1962).

Microorganisms could not be cultured from these soils and yet urease (McLaren et al., 1957; Skujiņš & McLaren, 1969), phosphatase (Skujiņš et al., 1962), and other enzymes (McLaren, 1969) remained active.

The practical implications and rationale for enzyme assays as soil health indicators was shown by Knight and Dick (2004). Using microwave irradiation to sterilize soils and isolate abiontic activity for GLU, they showed that differences in GLU activity due to soil management within the same soil type are due to the stabilized or abiontic fraction, not the activity associated with viable cells. This suggested that the abiontic fraction of GLU is not fixed but is a dynamic property that can be reduced by intensive land management. Its activity would, therefore, change steadily over time due to management with less chance for wide variability due to seasonal or environmental factors. Indeed, this conclusion is supported by the observation of Bandick and Dick (199) that short-term or in-season shifts in GLU activity are relatively stable.

Previous studies have used texture to control for enzyme activity for cross soil type comparisons using clay (Vallejo et al., 2010). However, for the sandy loams and loamy sands of NC with clay content at < 5% for many samples, control for sand were better accounted for the extent of activity complexed to the silt fraction of the soil. Activity controlled for sand resulted in no statistical difference between activity across the three physiographic regions in GLU_S (p = 0.3513). However, ARS_S had significantly higher activity at the Mountain site. This could be due to the higher proportion of no-till and organic treatments at the Mountain site (3/5) compared to the other two experiment stations (Carlson et al., 2015).

While there was no variation in soil type within the Piedmont and Mountain sites, the Coastal Plain site had changes in soil type and texture across the treatment plots. Control for sand in ARS (ARS_S) resulted in ranking changes. ARS ranked CTO1 (17.8 µg PNP g^{-1} hr^{-1}) below NTC (20.6

µg PNP g^{-1} hr^{-1}), whereas ARS_S ranked CTO1 (0.352 µg PNP g^{-1} hr^{-1} S^{-1}) above NTC (0.298 µg PNP g^{-1} hr^{-1} S^{-1}). Differences between treatments also became more pronounced with CTO2 activity almost double that of CTC for ARS but over triple for ARS_S. In GLU, the same trend was observed where CTO1, which received a lower ranking thank NTC for GLU, ranked higher for GLU_S. More importantly, where there was no significant difference between treatments in GLU, there was significant difference between treatments for GLU_S.

GLU_S and ARS_S had overall significant soils treatment effects (p = 0.0002, p = <0.0001) across the three sites. Treatments across the three sites can be generally categorized as those expected to have high (NTO), moderate (NTC, CTO1, CTO2, CTO, IRS, CPF, CPS), and low (CTC, DS, CPDF, CPDS, MPDF, MPDS, CTX) soil health based on the intensity of tillage and/or level of organic inputs. Using ratios of activity to sand resulted in a similar ranking of the soil management systems from high to low when ranked among all treatments. Across all experimental sites, NTO was significantly higher than the rest of the treatments for ARS_S (1.10 µg PNP g^{-1} hr^{-1} S^{-1}) and GLU_S (0.883 µg PNP g^{-1} hr^{-1} S^{-1}) (Table 2). Treatments expected to have moderate activity ranged from 17.8 – 47.2 µg PNP g^{-1} hr^{-1} and averaged 28.2 µg PNP g^{-1} hr^{-1} for ARS (Figure 3). For GLU, the activity ranged from 21.7 – 52.2 µg PNP g^{-1} hr^{-1} and averaged 31.9 µg PNP g^{-1} hr^{-1} (Figure 4). In contrast, standardizing to sand content the ARS_S ratios ranged from 0.298 – 0.858 µg PNP g^{-1} hr^{-1} S^{-1} and averaged 0.516 µg PNP g^{-1} hr^{-1} S^{-1}. CTO had the highest activity for ARS and ARS_S. The more intense tillage of CPF and CPS treatments ranked higher than the CTO. However, the ARS_S ratio revised this ranking. This latter ranking could logically make more sense, as the CTO treatment was receiving significant amounts of organic matter that should increase soil health.

For GLU_S, the ratios ranged from 0.349 - 0.899 µg PNP g^{-1} hr^{-1} S^{-1} and averaged 0.651 µg PNP g^{-1} hr^{-1} S^{-1}. NTC, a treatment at both the Coastal Plain and Mountain sites, did not fall into the same mid-range group; when controlled for sand, NTC fall into the same grouping at both sites. This is providing evidence that activity per unit texture ratios are giving results independent of soil type, because the Mountain site soil had over twice the clay content of the Coastal Plain soils. Across all sites, the most intensive tillage and chemical treatments had the lowest ranges in enzyme activity. Excluding the outlier of CPDS (ARS = 49.7 µg PNP g^{-1} hr^{-1}), ARS ranged from 11.4 – 26.7 µg PNP g^{-1} hr^{-1} and averaged 18.0 µg PNP g^{-1} hr^{-1}. Controlling for sand, ARS_S ranged from 0.160 – 0.484 µg PNP g^{-1} hr^{-1} S^{-1} and averaged 0.313 µg PNP g^{-1} hr^{-1} S^{-1}. For GLU, the activity range was 18.2 – 35.3 µg PNP g^{-1} hr^{-1} and averaged 25.3 µg PNP g^{-1} hr^{-1} and for GLU_S 0.257 - 0.608 µg PNP g^{-1} hr^{-1} S^{-1}, averaging 0.433 µg PNP g^{-1} hr^{-1} S^{-1}.

There is a clear trend wherein in conservation treatments receive higher average activity per unit sand than more intense treatments in ARS_S: conservation (1.10 µg PNP g^{-1} hr^{-1} S^{-1}) > moderate (0.516 µg PNP g^{-1} hr^{-1} S^{-1}) > intensive (0.313 µg PNP g^{-1} hr^{-1} S^{-1}) and GLU_S: conservation (0.883 µg PNP g^{-1} hr^{-1} S^{-1}) > moderate (0.651 µg PNP g^{-1} hr^{-1} S^{-1}) > intensive (0.433 µg PNP g^{-1} hr^{-1} S^{-1}). The maintenance of high variability within particularly “moderate” treatments is a useful quality of ARS_S and GLU_S because it allows for cross soil type comparisons without sacrificing sensitivity to subtly differing managements.

Comparison to CASH and HSHT

CASH and HSHT soil health indexes generally showed small differences in scoring and only detected significant soil management effects at one of the three sites. CASH scores ranged from 35 to 55 on a 0-100 scale. Two of the three sites showed no significant difference between treatments. HSHT evaluated two sites (Piedmont and Mountain). Three of nine treatments from

the Piedmont site were tested by the Roper team (2017) to represent a range of tillage: NTC, CPDS, and MPDS. The HSHT only found significant difference among treatments for the NTC site in the HSHT. CASH found no significant difference between the nine treatments. In contrast, GLU and ARS activity were able to significantly differentiate between NTC, CPDS, and MPDS with ARS having three distinct means groupings ($\alpha = 0.05$) for all nine treatments. Furthermore, rankings of GLU and ARS activities were able to detect the effect of spring vs. fall tillage and conservation vs. intensive tillage.

The HSHT found no significant difference between treatments for the mountain site, and CASH found two major groupings of treatment effects compared to three in both ARS and GLU activities. Additionally, the relative difference of scores for CASH is far less compared to ARS and GLU. CTC received a score of 48 for CASH and a score of only 49 for CTO. In comparison, CTC had less than half the activity of CTO for ARS and slightly over half the activity in GLU. The difference between the highest (NTO) and lowest (CTX/NTC) ranking for the Mountain site was 11 points on a 100-point scale with two treatments sharing the lowest ranking. ARS and GLU each had higher ranking of NTC than CTX, detecting subtle management differences. NTC had 2.7 times the activity of the CTC for ARS and 2.5 times for GLU. The higher activity in no-till treatments is consistent with other studies investigating the enzyme response to long-term tillage regimes (Dick, 1984; Deng & Tabatabai, 1996; Balota et al., 2004; Mbuthia et al., 2015).

CASH was not able to detect significant differences between treatments at the Coastal Plain site and had scores ranging from 46 for CTO2 to 38 for CTC. In contrast, ARS activity was able to detect treatment effects and had activities ranging from 21.3 µg PNP g^{-1} hr^{-1} for CTO2 to 11.4 µg PNP g^{-1} hr^{-1} for CTC. ARS_S maintained the same grouping and resulted in greater separation between CTO2 (0.520 µg PNP g^{-1} hr^{-1} S^{-1}) and CTC (0.160 µg PNP g^{-1} hr^{-1} S^{-1}). Controlling for

sand also resulted in significant treatment effects ($p < 0.05$) for GLU_S which held the same ranking as ARS_S with slightly differently ranked groupings. GLU_S had a higher percent difference between CTO2 (0.601 µg PNP g^{-1} hr^{-1} S^{-1}) and CTC (0.257 µg PNP g^{-1} hr^{-1} S^{-1}) than ARS_S. Given the heterogeneity of soil types at Goldsboro, controlling for sand allowed ARS_S and GLU_S to detect treatment effects across soil types.

Individual soil analyses of HSHT and CASH scoring that did show more detectable differences in soil management at the three North Carolina sites were mostly analyses related to soil biology, including soil protein and soil respiration (Roper et al., 2017). However, respiration varies widely on a seasonal basis, making it difficult to calibrate. Enzymes with a significant amount of abiontic activity have been shown the ability to be seasonally stable and detect subtle management practices (Bandick & Dick, 1999; Knight & Dick, 2004). Thus, future refinement and development of soil health tests should include arylsulfatase and β-glucosidase, as these have a significant amount of abiontic activity.

Enzyme Activity and Microbial Community

The NTC treatment of the Piedmont had the highest concentrations of bacterial, fungal, and total FAMEs of the treatments (Figure 7). Tillage breaks up aggregates, thereby destroying soil habitat and reducing pore space for adequate air and water flow (Gupta & Germida, 1988). The somewhat significant correlation between GLU and these FAMEs reflects how destruction of soil habitat leading to reduced microbial community populations can lead to reduced enzyme production. ARS had a significant relation with AMF and total fungi. Since fungi are hyphal, tillage breaks apart hyphae and weakens or kills fungi in the process. This was shown by the data from the Mountain site wherein the highest fungal FAMEs are in the no-till treatments (Figure 6). Fungi are eukaryotic in comparison to prokaryotic bacteria, making them more susceptible to

eukaryotic-targeted herbicide, insecticide, and fungicides, which is shown at the Coastal Plain site. Here bacterial FAMEs increased from CTC to NTC, but total fungal FAMEs actually decreased (Figure 5). Additionally, at this site fungi and bacteria responded positively to the presence of cover crops in CTO2 compared to CTO1. Cover crops like the rye used in CTO2 have root systems reaching deep within the soil. These roots release polysaccharides which stimulate the microbial community and, upon crop termination, are available for decomposition

Fatty acid methyl esters as biomarkers for microbial groups were correlated with ARS, GLU, ARS_S, and GLU_S. The highest correlation between activity and FAME was with the arbuscular mycorrhizal fungi (AMF) marker and ARS ($r = 0.34$, $p = 0.0065$) followed by total fungi and ARS ($r = 0.31$, $p = 0.0127$) (Table 3). This is consistent with the established relationship of ARS with living fungal biomass (Li & Sarah, 2003). While both fungi and bacteria produce arylsulfatases, only fungi contain its substrate, ester sulfates, and in turn microbial communities produce more sulfatases. Thereby, increased fungal populations would increase substrate availability. GLU had the highest correlation with AMF ($r = 0.27$, $p = 0.0411$) and total fungi ($r = 0.27$, $p = 0.0309$). Correlations between biomarkers and activity were nearly identical for uncontrolled and textural controlled enzyme activities. ARS and GLU both had a correlation trend with total FAMEs, where each had a correlation coefficient of 0.24 ($p = 0.055$). ARS had a significant correlation with $Gram^-$ ($r = 0.28$, $p = 0.00249$) and $Gram^+$ ($r = 0.18$, $p = 0.0163$) bacteria while GLU had a somewhat significant correlation with $Gram^+$ bacteria ($r = 0.23$, $p = 0.0627$) and no significant correlation with $Gram^-$. The trend of stronger correlations of ARS to microbial biomass than GLU can be explained by the higher proportion of GLU enzymes in the abiontic fraction compared to ARS (Knight & Dick, 2004). These results provide a mechanistic interpretation for using enzyme activities as soil health indicators as they are related to microbial

community. However, its an integrative measure as a variety of microorganisms produce these enzymes, and the abiontic fraction represents the cumulative microbial activity leading up to the time of sampling.

CONCLUSIONS

Enzyme activity detected significant difference between treatments at all three sites when controlled for sand. Both GLU_S and ARS_S had a significantly positive response to poultry manure and cover crop amendments. Enzyme activity had a significant positive response to no-till management in two of the three sites and consistently had higher activity in spring tillage plots compared to fall tillage at the Piedmont. ARS_S and GLU_S had a significant negative response to intensive tillage in the form of conventional tillage at the Mountain and Coastal Plain site and the MPDS and MPDF at the Piedmont. Enzyme activity controlled for sand detected significant treatment effects across all three sites for both enzymes and resulted in a change in ranking at the Coastal Plain site. Additionally, activity controlled for sand better ranked best management practices. For example, CPF and CPS were ranked higher than the Mountain CTO but lower than CPF and CPS for ARS_S, following the trend of higher enzyme activity in treatments with organic compared to chemical amendments. For both enzymes, NTO had significantly higher enzyme activity across all three sites, showing the significant increase in activity of combined no-till and organic management.

These results indicate the potential of arylsulfatase and β-glucosidase enzyme assays to serve as a viable soil health metric. This metric can be incorporated into existing soil health indexes, replacing or assigning higher weight than metrics which don't detect differences between treatments or cannot control for soil type. Additionally, these results further support the positive impact of no-till and organically managed systems on the soil microbial community.

Future work should further validate the use of sand as a control for texture compared to other controls for soil type such as organic matter.

TABLES AND FIGURES

TABLE 1 Summary of abbreviations of treatments for all three research sites.

Location	Abbreviation	Treatment	pH	Sand %	Clay %
Goldsboro (Coastal Plain)	NTC	No-till chemical	6.2	68	5
	CTC	Conventional tillage chemical	6.2	72	6
	CTO1	Conventional tillage organic 1	6.8	63	13
	CTO2	Conventional tillage organic 2	6.1	48	6
Reidsville (Piedmont)	NTC	No-till chemical	5.7		
	IRS	In-row subsoiling	5.9		
	DS	Spring disking	5.9		
	CPF	Fall chisel plowing	6.1		
	CPS	Spring chisel plowing	6.0	58	17
	CPDF	Chisel plowing and fall disking	5.9		
	CPDS	Chisel plowing and spring disking	5.8		
	MPDF	Moldboard plowing and fall disking	6.0		
	MPDS	Moldboard plowing and spring disking	6.0		
Mills River (Mountain)	NTC	No-till chemical	6.3		
	NTO	No-till organic	6.3		
	CTC	Conventional tillage chemical	6.0	55	19
	CTO	Conventional tillage organic	6.5		
	CTX	Conventional tillage control	6.4		

TABLE 2 Comparison of Cornell Soil Health Assessment (CASH) soil health scores, Haney Soil Health Test soil health scores, ARS, GLU, ARS_S, and GLU_S by location. Lowercase letters indicate means separation within site for ARS and GLU and across sites for ARS_S and GLU_S (α=0.05).

Location	Treatment	CASH	HSHT	ARS	GLU	ARS_S	GLU_S
				-----µg PNP g^{-1} hr^{-1}-----		----------µg PNP g^{-1} hr^{-1} S^{-1}----------	
Goldsboro (Coastal Plain)	NTC	45	-	20.6ab	24.2	0.298efg (ab)	0.349de (b)
	CTC	38	-	11.4b	18.4	0.160g (b)	0.257e (b)
	CTO1	43	-	17.8ab	23.4	0.352bcdef (ab)	0.479abcd (ab)
	CTO2	46	-	21.3a	26.0	0.520ab (a)	0.601ab (a)
Reidsville (Piedmont)	NTC	43	16a	27.3ab	37.6bc	0.469efg	0.647abcd
	IRS	46	-	28.4a	35.3bc	0.490bcdef	0.608abcd
	DS	46	-	18.5abc	24.8c	0.320def	0.422cdef
	CPF	46	-	24.5abc	38.0b	0.422cdef	0.654abc
	CPS	46	-	29.2a	52.2a	0.504bcdef	0.899a
	CPDF	45	-	19.9abc	35.3bc	0.344def	0.608abcd
	CPDS	38	8b	49.7a	33.8bc	0.856abcde	0.583abcd
	MPDF	35	-	13.6bc	28.7bc	0.235efg	0.494cdef
	MPDS	39	5b	13.8c	24.2c	0.238fg	0.416cde
Mills River (Mountain)	NTC	44b	12	38.0ab	21.7bc	0.690abcd	0.395de
	NTO	55a	21	60.4a	48.5a	1.10a	0.883a
	CTC	48ab	7	22.5c	19.1c	0.410bcdef	0.346cde
	CTO	49ab	19	47.2a	33.5ab	0.858abc	0.611abc
	CTX	44b	8	26.7bc	18.2c	0.484bcdef	0.330cde

Note. Groupings in parentheses represent within site separation.

TABLE 3 Correlation coefficients (*r*-values) for FAME biomarkers and enzyme activities with *p* in parentheses.

	Gen. gram	Gram-	AMF	Saprophytic fungi	Total Gram+	Total bacteria	Total fungi	Total FAME
ARS	0.17^{ns}	0.28*	0.34**	0.28*	0.18*	0.21^{ns}	0.31*	0.24^{ns}
GLU	0.25*	0.17^{ns}	0.27*	0.26*	0.23^{ns}	0.23^{ns}	0.27*	0.24^{ns}
ARS$_S$	0.17^{ns}	0.29*	0.32**	0.29*	0.18^{ns}	0.21^{ns}	0.31*	0.24*
GLU$_S$	0.25*	0.26*	0.25*	0.27*	0.23^{ns}	0.24^{ns}	0.28*	0.25*

^{ns} Not significant at $p < 0.05$

* $p < 0.05$

** $p < 0.01$

FIGURE 1 Arylsulfatase enzyme activity across three NC physiographic regions by treatment.

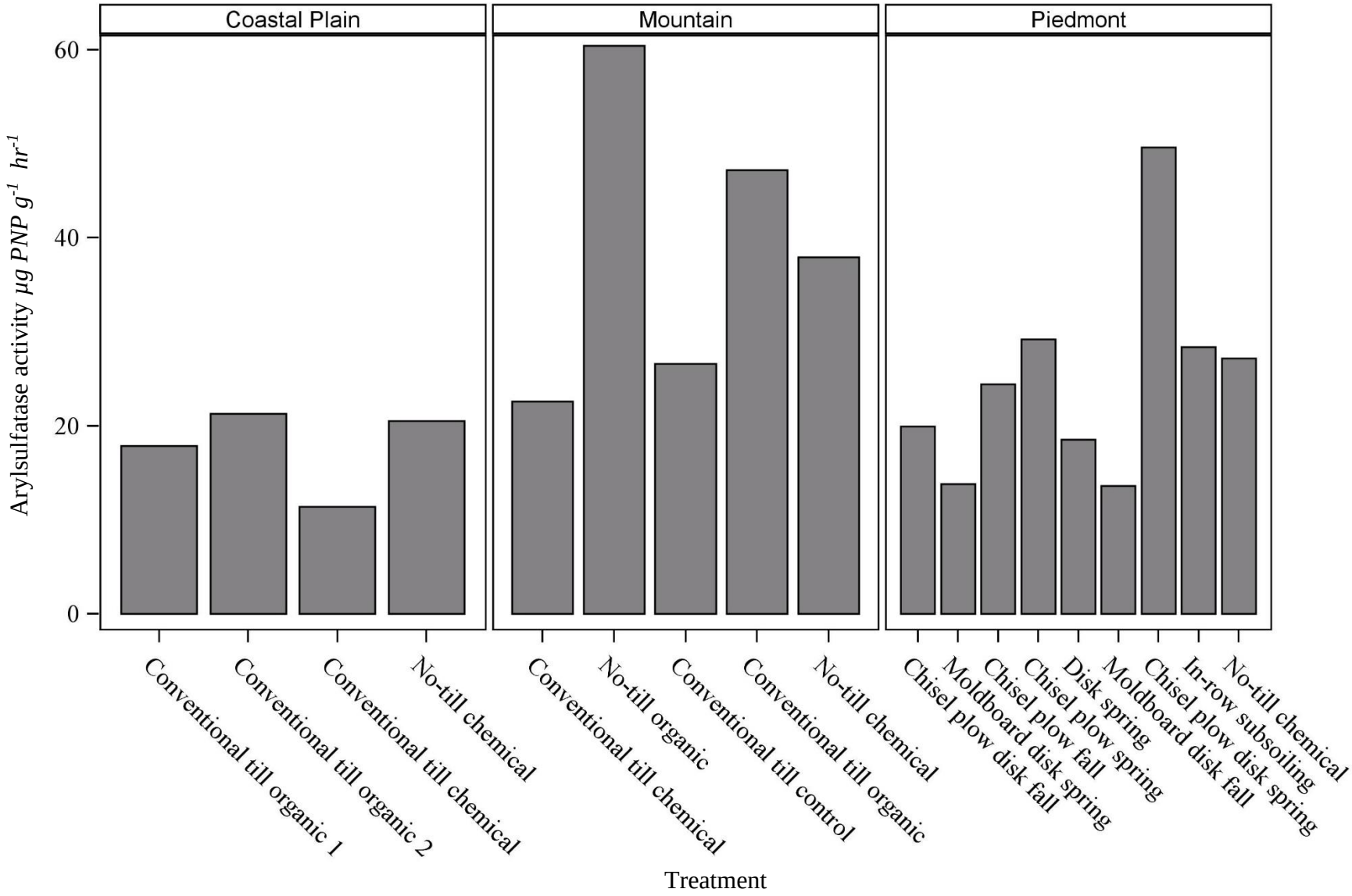


FIGURE 2 B-glucosidase enzyme activity across three NC physiographic regions by treatment.

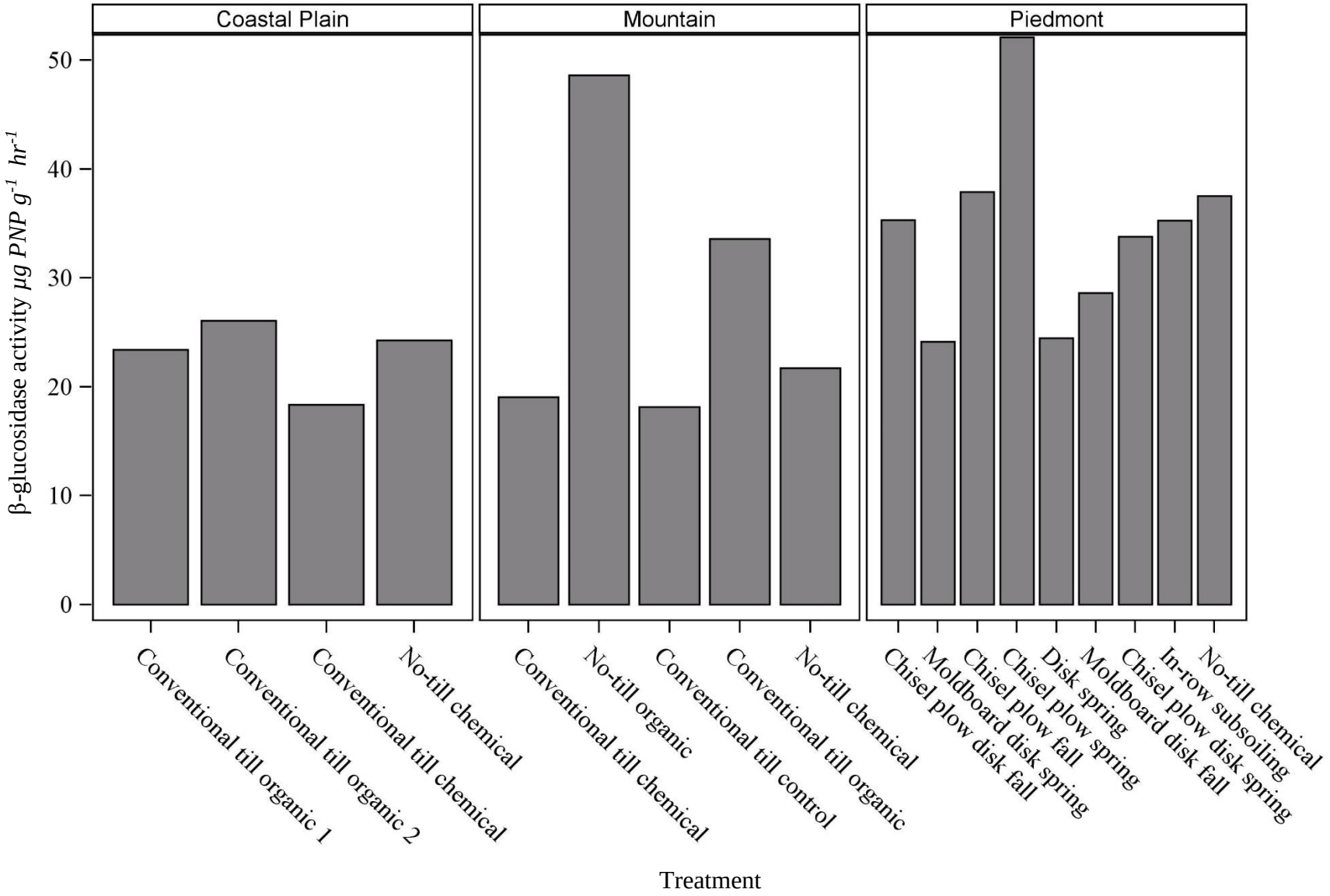


FIGURE 3 Arylsulfatase activity per unit sand across three NC physiographic regions by treatment.

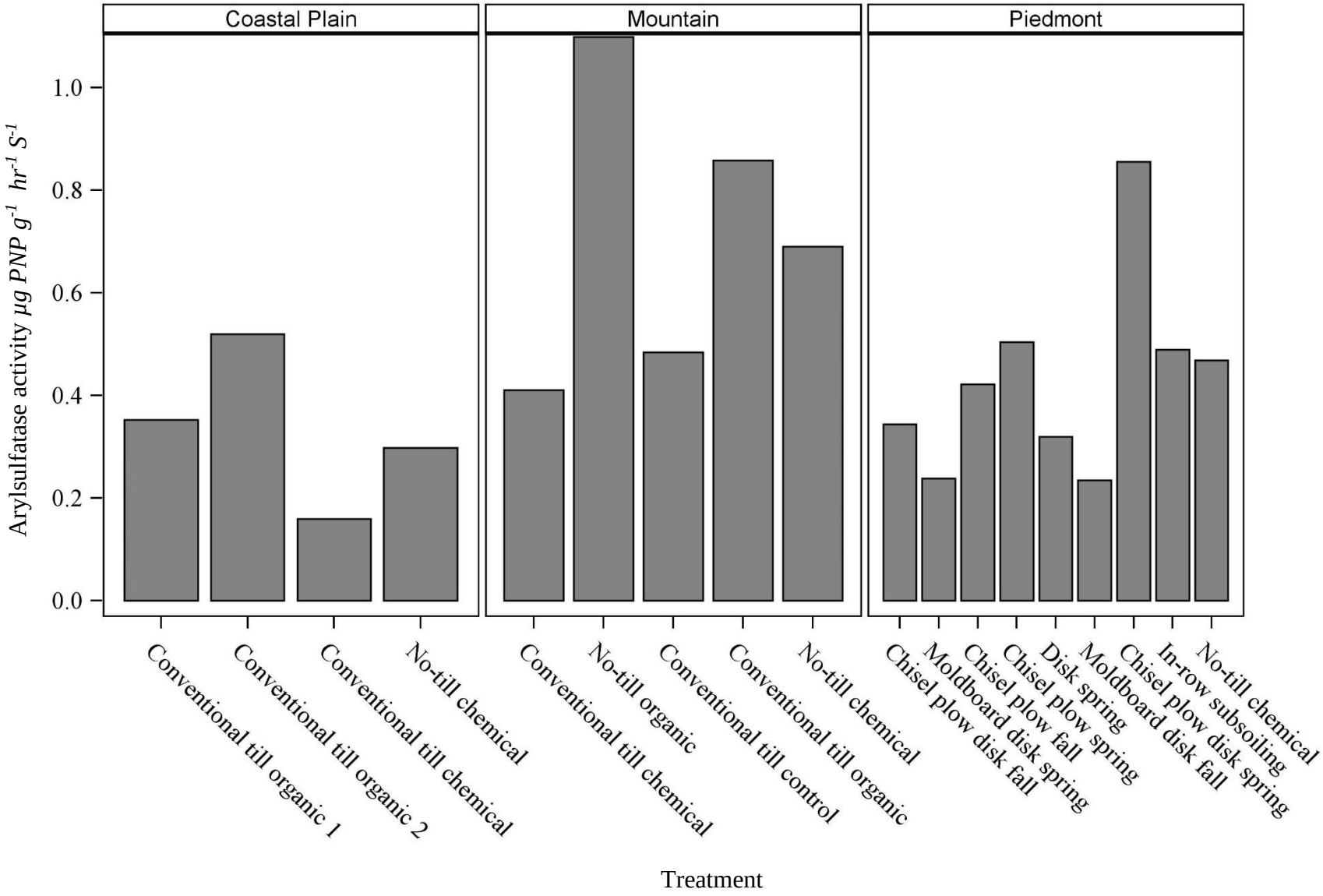


FIGURE 4 B-glucosidase activity per unit sand across three physiographic regions by treatment.

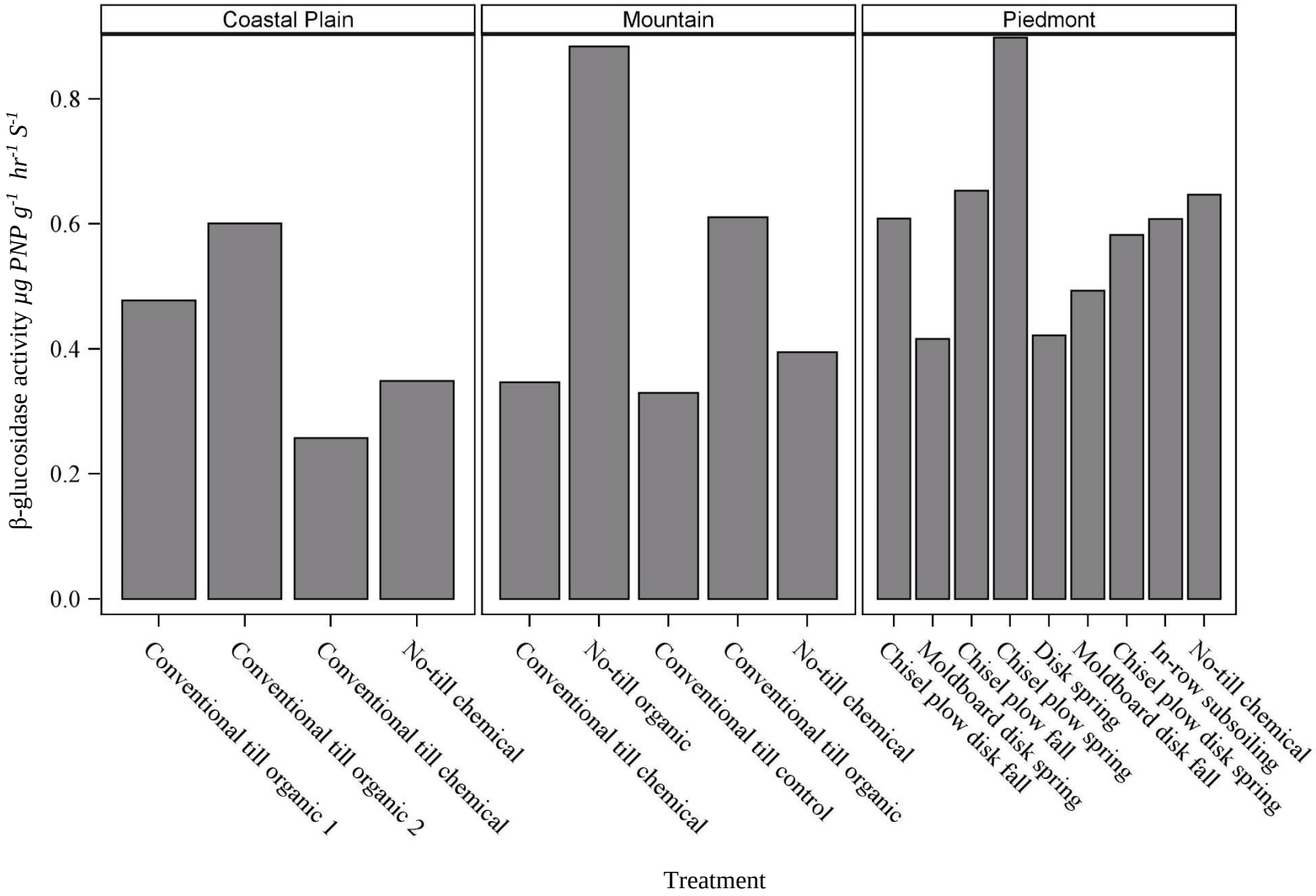


FIGURE 5 FAME concentrations by treatment at that Coastal Plain site (Goldsboro, NC).

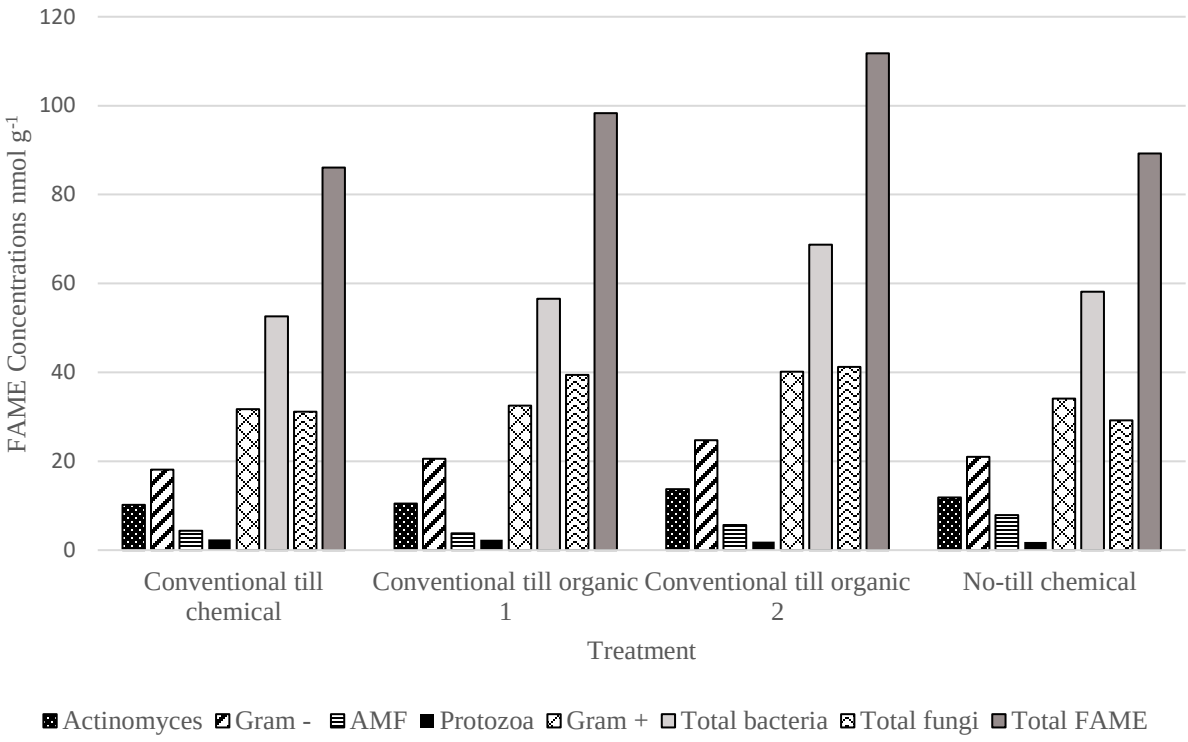


FIGURE 6 FAME concentrations by treatment at the Mountain site (Mills River, NC).

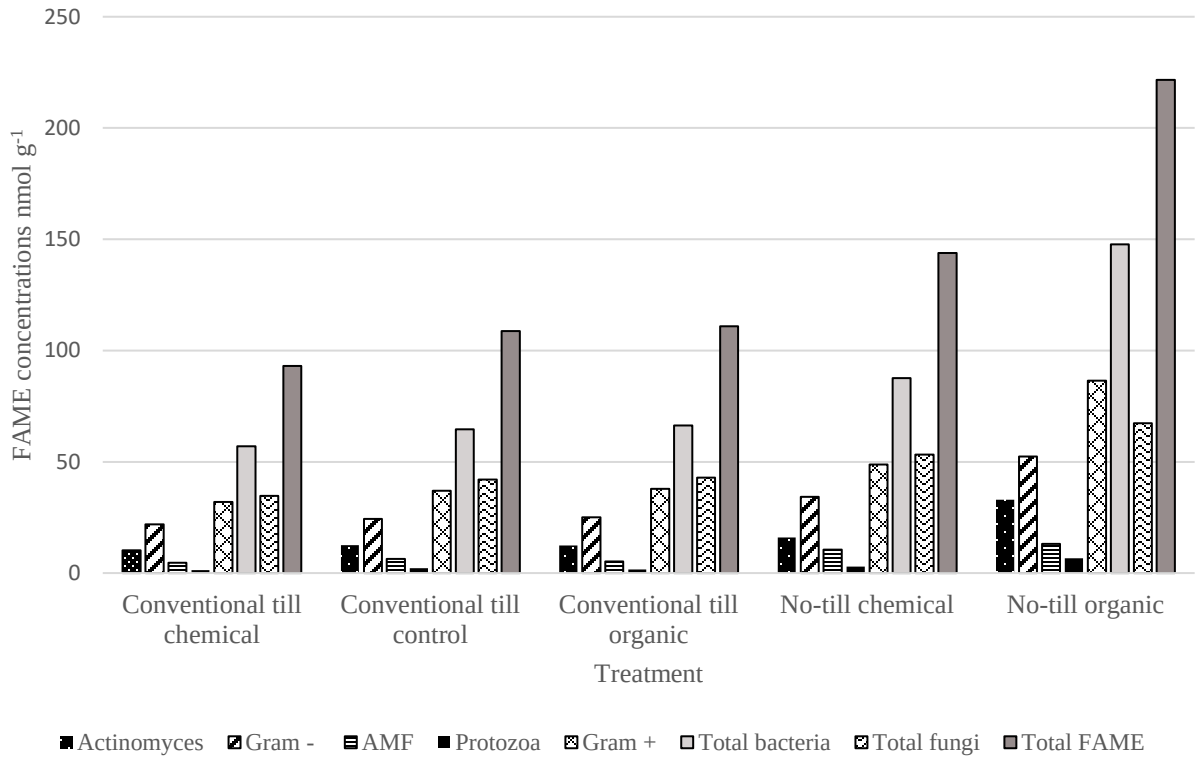
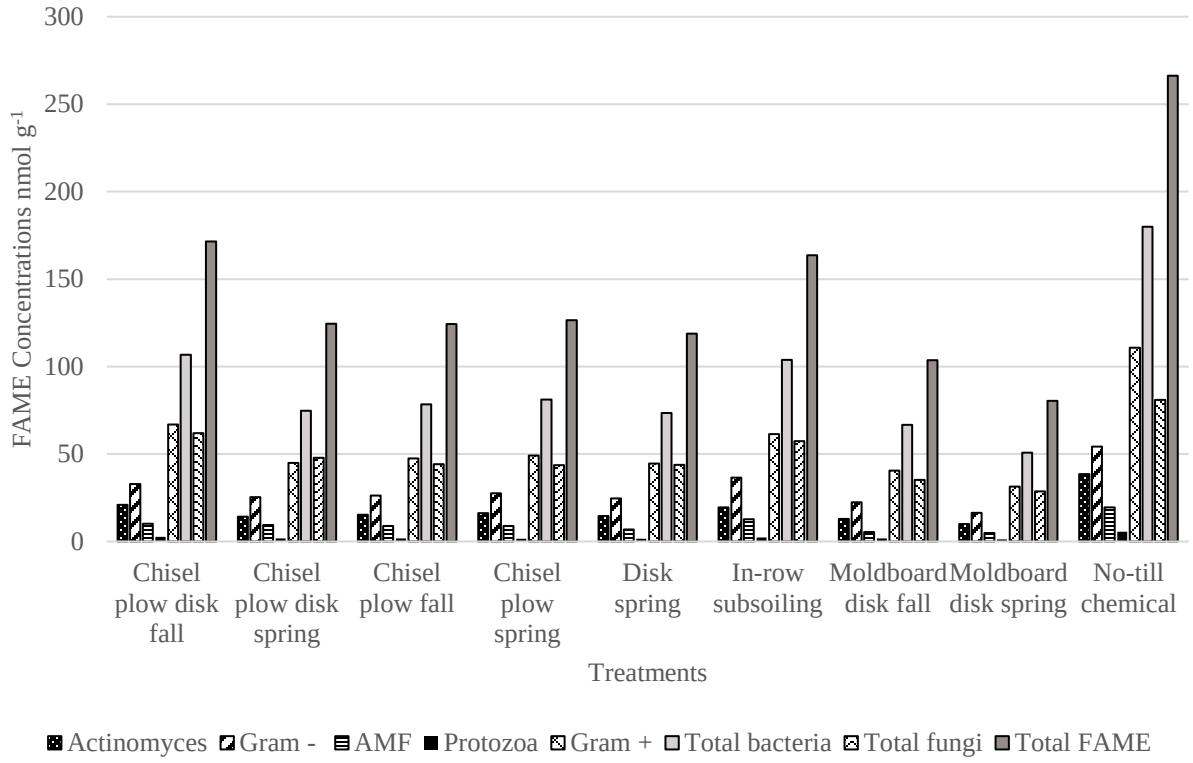


FIGURE 7 FAME concentrations by treatment at the Piedmont site (Reidsville, NC).



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REFERENCES

Acosta-Martinez, V., Moore-Kucera, J., Cotton, J., & Gardner, T. (2013). Soil enzyme activities during the 2011 Texas record drought/heatwave and implications to biogeochemical cycling and organic matter dynamics. *USDA-ARS / UNL Faculty*, 1414.

Acosta-Martinez, V., Perez-Guzman, L., & Johnson, J. M. F. (2019). Simultaneous determination of β-glucosidase, β-glucosaminidase, acid phosphomonoesterase, and arylsulfatase activities in a soil sample for a biogeochemical cycling index. *Applied Soil Ecology, 142*, 72-80.

Acosta-Martinez, V., Zobeck, T. M., Gill, T. E., & Kennedy, A. C. (2003). Enzyme activities and microbial community structure in semiarid agricultural soils. *Biology and Fertility of Soils, 38*, 216-227.

Balota, E. L., Kanashiro, M., Fihlo, A. C., Andrade, D. S., & Dick, R. P. (2004). Soil enzyme activities under long-term tillage and crop rotation systems in subtropical agroecosystems. *Brazilian Journal of Microbiology, 35*, 300-306.

Balota, E. L., Yada, I. F., Amaral, H., Nakatani, A. S., Dick, R. P., & Coyne, M. S. (2014). Long-term land use influences soil microbial biomass P and S, phosphatase and arylsulfatase activities, and S mineralization in a Brazilian Oxisol. *Land Degradation & Development, 25*, 397-406.

Bandick, A. K., & Dick, R. P. (1999). Field management effects on soil enzyme activities. *Soil Biology and Biochemistry, 3*1, 1471-1479.

Bossio, D. A., & Scow, K. M. (1998). Impacts of carbon and flooding on soil microbial

communities: Phospholipid fatty acid profiles and substrate utilization patterns. *Microbial Ecology, 35*, 265-278.

Bouyoucos, G. J. (1962). Hydrometer method improved for making particle size analysis of soils. *Agronomy Journal. 54*, 464-465.

Boyd, S. A., & Mortland, M. M. (1990). Enzymes interactions with clays and clay-organic matter complexes. *Soil Biochemistry, 6*.

Burns, R. G. (1978). *Soil Enzymes*. New York, NY: Academic Press.

Carlson, J., Saxena, J., Basta, N., Hundal, L., Busalacchi, D., & Dick, R. P. (2015). Application of organic amendments to restore degraded soil: Effects on soil microbial properties. *Environmental Monitoring and Assessment, 187*(3), 109.

Cespedes, M. C., Stone, A., & Dick, R. P. (2006). Organic soil amendments: Impacts on snap bean common root rot (*Aphanomyes euteiches*) and soil quality. *Applied Soil Ecology, 31*, 199-210.

Cornell Soil Health Team. (2018). *Comprehensive Assessment of Soil Health*. Retrieved from http://soilhealth.cals.cornell.edu/

Darby, H. M., Stone, A. G., & Dick, R. P. (2006). Compost and manure mediated impacts on soilborne pathogens and soil quality. *Soil Science Society of America Journal, 70*, 347-358.

Deng, S., & Popova, I. (2011). Carbohydrate hydrolases. In R. P. Dick (Ed.), *Methods of Soil Enzymology, Volume 9* (319-369). Madison, WI: SSSA https://doi.org/10.2136/sssabookser9.c9

Deng, S. P., & Tabatabai, M. A. (1996). Effect of tillage and residue management on enzyme activities in soils. II. Glycosidases. *Biology and Fertility of Soils, 22*, 208-213.

Deng, S. P. & Tabatabai, M. A. (1997). Effect of tillage and residue management on enzyme activities in soil. III. Phosphatases and arylsulfatase. *Biology and Fertility of Soils, 24*, 141-146.

Dick, R. P., Rasmussen, P. E., & Kerle, E. A. (1988). Influence of long-term residue management on soil enzyme activity in relation to soil chemical properties of a wheat-fallow system. *Biology and Fertility of Soils, 6*, 159-164.

Dick, R. P. (1992). A review: Long-term effects of agricultural systems on soil biochemical and microbial parameters. *Agriculture, Ecosystems, and Environment, 40*, 25-36.

Dick, R. P. (1994). Soil enzyme activities as indicators of soil quality. *Defining Soil Quality for a Sustainable Environment, 35*, 107-124.

Dick, R. P. (1997). Soil enzyme activities as integrative indicators of soil health. In C.E. Pankhurst, B.M. Doube, V.V.S.R. Gupta (Eds.), *Biological Indicators of Soil Health* (121-156). Wallingford: CAB International.

Dick, W. A. (1984). Influence of long-term tillage and crop rotation combination on soil enzymology. *Soil Science Society of America Journal, 48*, 569-574.

Doran, J. W., & Parkin, T. (1994). Defining and assessing soil quality. In J. Doran, D. Coleman, D. Bezdicek, & B. Stewart (Eds.), *Defining soil quality for a sustainable environment*. Minneaspolis, MN: SSSA Special Publications.

Dunn, C. G., Campbell, W. L., Fram, H., & Hutchins, A. (1948). Biological and photochemical effects of high energy, electrostatically produced Roentgen rays and cathode rays. *Journal of Applied Physics, 19,* 605-616.

EPA. (2004). Soil and waste pH. Method 9045D. SW-846 Methods. Test methods for evaluating solid waste, physical/chemical methods.

Eivazi, F., Bayan, M., & Schmidt, K. (2003). Select soil enzyme activities in the historic Sanborn Field as affected by long-term cropping systems. *Communications in Soil Science and Plant Analysis, 34*, 2259-2275.

Farrell, R. E., Gupta, V. V. S. R., & Germinda, J. J. (1994). Effects of cultivation on the activity and kinetics of arylsulfatase in Saskatchewan soils. *Soil Biology and Biochemistry, 26*, 1033-1040.

Geisseler, D., & Scow, K. M. (2014). Long-term effects of mineral fertilizers on soil microorganisms—A review. *Soil Biology and Biochemistry, 75*, 54-63.

Groffman, P. M., Eagan, P., Sullivan, W. M., & Lemunyon, J. L. (1996). Grass species and soil type effects on microbial biomass and activity. *Plant and Soil, 183*, 61-67.

Gugina, B. K., Idowu, O. J., Schindelbeck, R. R., van Es, H. M., Moebius-Clune, B. N., Wolfe, D. W., … & Abawi, G. S. (2009). Cornell Soil Health Assessment Training Manual, Edition 2.0. Cornell University, Geneva, NY.

Gupta, V. V. S. R., & Germida, J. J. (1988). Distribution of microbial biomass and its activity in different soil aggregate size classes as affected by cultivation. *Soil Biology & Biochemistry, 20*(6), 777-786.

Haig, A. D. (1955). Some characteristics of esterase- and urease-like activity in the soil. Ph.D. diss. University of California, Davis.

Hinojosa, M. B., Carreira, J. A., Garcia-Ruiz, R., & Dick, R. P. (2004). Soil moisture pre-treatment effects on enzyme activities as indicators of heavy metal contaminated and reclaimed soil. *Soil Biology and Biochemistry, 36*, 1559-1568.

Hoffmann, G. (1959). Distribution and origin of some enzymes in soil. *Z. Pflanzenernähr. Düng. Bodenk, 85*, 97-104.

Ibekwe, A. M., & Kennedy, A. C. (1998). Phospholipid fatty acid profiles and carbon utilization patterns for analysis of microbial community structure under field and greenhouse conditions. *Microbiology Ecology, 26*(2), 151-163.

Inagaki, T. M., Moraes Sa, J. C., Caires, E. F., & Potma Goncalves, D. R. (2016). Lime and gypsum applications increase biological activity, carbon pools, and agronomic productivity in highly weathered soil. *Agriculture, Ecosystems & Environment, 231*, 156-165.

Jian, S., Li, J., Chen, J., Wang, G., Mayes, M. A., Dzantor, K.E., … & Lou, Y. (2016). Soil extracellular enzyme acticities, soil carbon and nitrogen storage under nitrogen fertilization: A meta-analysis. *Soil Biology and Biochemistry, 101*, 32-43.

Knight, T. R., & Dick, R. P. (2004). Differentiating microbial and stabilized β-glucosidase activity relative to soil quality. *Soil Biology and Biochemistry, 36,* 2089-2096.

Kooch, Y., Ehsani, S., & Akbarinia, M. (2019). Stoichiometry of microbial indicators shows clearly more soil responses to land cover changes than absolute microbial activities. *Ecological Engineering, 131*, 99-106.

Lee, Y. B., Lorenz, N., Kincaid Dick, L. K., & Dick, R. P. (2007). Cold storage and pretreatment incubation: Effects of soil microbial properties. *Soil Science Society of America Journal, 71*, 1299-1305.

Li, X., & Sarah, P. (2003). Arylsulfatase activity of soil microbial biomass along a Mediterranean-arid transect. *Soil Biology and Biochemistry*, *35*(7), 925-934.

Mbuthia, L. W., Acosta-Martinez, V., DeBruyn, J., Schaeffer, S., Tyler, D., Odoi, E., … & Eash, N. (2015). Long term tillage, cover crop, and fertilization effects of microbial community structure, activity: Implications for soil quality. *Soil Biology & Biochemistry, 89*, 24-34.

McLaren, A. D. (1969). Radiation as a technique in soil biology and biochemistry. *Soil Biology and Biochemistry, 1*, 63-73.

McLaren, A. D., & Packer, L. (1970). Some aspects of enzyme reactions in heterogeneous systems. *Advanced Enzymology, 33*, 245-308.

McLaren, A. D., Reshetko, L., & Huber, W. (1957). Sterilization of soil by irradiation with an electron beam, and some observation on soil enzyme activity. *Soil Science, 83*, 497-502.

McLaren, A. D., Luse, R. A., & Skujiņš, J. J. (1962). Sterilization of soil by irradiation and some further observation on soil enzyme activity. *Soil Science Society of America Proceedings, 26*, 371-377.

Mehlich, A. (1948). Photometric determination of humic matter in soils, proposed method. *Communications in Soil Science and Plant Analysis, 15*, 1417-1422.

Mendes, I. C., Souza, L. M., Sousa, D. M. G., Lopes, A. A. C., Reis-Junior, F. B., Coelho Lacerda, M. P., & Vitoria Malaquias, J. (2019). Critical limits for microbial indicators in tropical Oxisols at post-harvest: The FERTBIO soil sample concept. *Applied Soil Ecology, 139*, 85-93.

Mganga, K. Z., Razavi, B. S., & Kuzyakov, Y. (2015). Microbial and enzymes response to nutrient additions in soil of Mt. Kilimanjaro region depending on land use. *European Journal of Soil Biology, 69*, 33-40.

Miller, M., & Dick, R. P. (1995). Dynamics of soil C and microbial biomass on whole soil and aggregates in two cropping systems. *Applied Soil Ecology, 2*, 253-261.

Moraes Sa, J. C., Potma Goncalves, D. R., Ferreira, L. A., Mishra, U., Inagaki, T. M., Ferreira Furlan, F. J., … & Oliveira Ferreira, A. (2018). Soil carbon fractions and biological

activity based indices can be used to study the impact of land management and ecological successions. *Ecological Indicators, 84*, 96-105.

Nannipieri, P., Trasar-Cepeda, C., & Dick, R. P. (2018). Soil enzyme activity: A brief history and biochemistry as a basis for appropriate interpretations and meta-analysis. *Biology and Fertility of Soils, 54*(1), 11-19.

Nunes, M. R., van Es, H. M., Schindelbeck, R., Ristow, A. J., & Ryan, M. 2018. No-till and cropping system diversification improve soil health and crop yield. *Geoderma, 328*, 30-43.

Ochiai, N., Powelson, M. L., Crowe, F. J., & Dick, R. P. (2008). Green manure effects on soil quality in relation to suppression of Verticillium wilt of potatoes. *Biology and Fertility of Soils, 44*, 1013-1023.

Quiquampoix, H., Servagent-Noinville, S., & Baron, M. H. (2002). Enzyme adsorption on soil mineral surfaces and consequences for the catalytic activity. *Enzymes in the environment. Marcel Dekker, New York*, 285-306.

Raper, R. L. (2002). The influence of implement type, tillage depth, and tillage timing on residue burial. *American Society of Agricultural Engineers, 45*(5), 1281-1286.

Roper, W. R., Osmond, D. L., Heitman, J. L., & Reberg-Horton, S. C. (2017). Soil health indicators do not differentiate among agronomic management systems in North Carolina soils. *Soil Science Society of America Journal, 81*, 828-843.

Roper, W. R., Osmond, D. L., & Heitman, J. L. (2019). A response to "Reanalysis validates soil health indicator sensitivity and correlation with long-term crop yields." *Soil Science Society of America Journal, 83*(6), 1842-1845.

Schutter, M. E., & Dick, R. P. (2000). Comparison of fatty acid methyl ester (FAME) methods

for characterizing microbial communities. *Soil Science Society of America Journal, 64*, 1659-1668.

Sharma, P., Singh, G., & Singh, R. P. (2013). Conservation tillage and optimal water supply enhance microbial enzyme (glucosidase, urease and phosphatase) activities in fields under wheat cultivation during various nitrogen management practices. *Archives of Agronomy and Soil Science, 59*, 911-928.

Skujiņš, J. J., Braal, L., & McLaren, A. D. (1962). Characterization of phosphatase in a terrestrial soil sterilized with an electron beam. *Enzymologia, 25*, 125-133.

Skujiņš, J. J., & McLaren, A. D. (1969). Assay of urease activity using ^{14}C-urea in stored, geologically preserved soils. *Soil Biology and Biochemistry, 1*, 88-99.

Sun, Z., Bruun, E. W., Arthur, E., Jonge, L. W., Moldrup, P., Hauggard-Nielsen, H., & Elsgaard, L. (2014). Effect of biochar on aerobic processes, enzymatic activity, and crop yields in two sandy loam soil. *Biology and Fertility of Soils, 50*, 1087-1097.

Tabatabai, M. A. (1994). Soil enzymes. In R. W. Weaver, Angle, S., Bottomley, P., Bezdicek, D., Smith, S., Tabatabai, A., & Wollum, A (Eds.), *Methods of Soil Analysis: Part 2 Microbiological and Biochemical Properties, 5.2.* (775-833). Madison, WI: SSSA http://dx.doi.org/10.2136/sssabookser5.2

Turner, B. L., Hopkins, D. W., Haygarth, P. M., & Ostle, N. (2002). β-Glucosidase activity in pasture soils. *Applied Soil Ecology, 20*, 157-162.

USDA-NRCS. (2015). Soil health. Retrieved from https://www.nrcs.usda.gov/wps/portal/nrcs/main/soils/health/

Vallejo, V. E., Roldan, F., & Dick, R. P. (2010). Soil enzymatic activities and microbial biomass in an integrated agroforestry chronosequence compared to monoculture and a native

forest of Colombia. *Biology and Fertility of Soils, 46*, 577-587.

Van Es, H. M., & Karlen, D. L. (2019). Reanalysis validates soil health indicator sensitivity and correlation with long-term crop yields. *Soil Science Society of America Journal, 83*, 721-723.

Veum, K. S., Kremer, R. J., Sudduth, K. A., Kitchen, N. R., Lerch, R. N., Baffaut, C., … & Sadler, E. J. (2015). Conservation effects on soil quality indicators in the Missouri Salt River Basin. *Journal of Soil and Water Conservation, 70*, 232-246.

Wallenius, K., Rita, H., Mikkonen, A., Lappi, K., Lindstrom, K., Hartikainen, H., … & Niemi, R. M. (2011). Effects of land use on the level, variation, and spatial structure of soil enzyme activities and bacterial communities. *Soil Biology and Biochemistry, 43*, 1464-1473.

Ward Laboratories, Inc. (2018). *Haney Soil Health Test Information Rev. 1.0*. Retrieved from https://www.wardlab.com/haney-info.php

Yang, X., & Wander, M. M. (1998). Temporal changes in dry aggregate size and stability: Tillage and crop effects on a silty loam Mollisol in Illinois. *Soil and Tillage Research, 49*(3), 173-183.